Characterization and turnover of CD73/IP(3)R3-positive microvillar cells in the adult mouse olfactory epithelium

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Abstract: The main olfactory epithelium consists of 4 major cell types: sensory neurons, supporting cells, microvillar cells, and basal progenitor cells. Several populations of microvillar olfactory cells have been described, whose properties are not yet fully understood. In this study, we aimed to clarify the classification of microvillar cells by introducing a specific marker, CD73. Furthermore, we investigated the turnover of CD73-microvillar cells during adult life. Using direct and indirect immunofluorescence in adult main olfactory epithelium, we first demonstrate that ecto-5'-nucleotidase (CD73) is a reliable marker for microvillar cells reported previously to express phospholipase C 2 (PLC 2) along with type 3 IP(3) receptors (IP(3)R3) and transient receptor potential channels 6 (TRPC6), as well as for cells labeled by transgenic expression of tauGFP driven by the IP(3)R3 promoter. The ubiquitous CD73 immunoreactivity in the microvilli of these 2 cell populations indicates that they correspond to the same cell type (CD73-microvillar cell), endowed with a signal transduction cascade mobilizing Ca(++) from intracellular stores. These microvillar cells respond to odors, possess a basal process, and do not degenerate after bulbectomy, suggesting that they contribute to cellular homeostasis in the olfactory epithelium. Next, we examined whether CD73-microvillar cells undergo turnover in the adult olfactory epithelium. By combining CD73 immunofluorescence and BrdU pulse labeling, we show delayed BrdU incorporation in a small fraction of CD73-positive microvillar cells, which persists for several weeks after BrdU administration. These findings indicate that CD73-microvillar cells likely differentiate from proliferating progenitor cells and have a slow turnover despite their apical position in the olfactory epithelium. These combined properties are unique among olfactory cells, in line with the possibility that they might regulate cellular homeostasis driven by extracellular ATP and adenosine.

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Origin and turnover of microvillar cells in the adult mouse olfactory epithelium

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Abstract

The olfactory epithelium is directly exposed to the airborne environment; consequently most olfactory cells have a limited lifespan and are continuously replaced in adult animals. Olfactory sensory neurons derive from a pool of progenitor cells localized in the basal germinative zone. In contrast, the supporting cells forming the apical layer of the neuroepithelium have the capability to self-renew. The aim of the present study was to investigate whether microvillar cells (MVCs), a major population of microvilli-bearing cells in the olfactory epithelium, undergo a similar turnover. MVCs are a specialized cell type endowed with inositol-triphosphate ($InsP_3$)-mediated signal transduction cascade. They are evenly distributed at the surface of the epithelium and express neuropeptide Y, a neurotransmitter regulating stem cell proliferation. Therefore, MVCs might contribute to maintain neuronal homeostasis in the olfactory epithelium. Here, we identified ecto-5'-nucleotidase (CD73) as a specific marker for MVCs in mice. By using CD73 immunofluorescence combined with BrdU pulse-labeling, we observed that MVCs have a slow turnover considering their apical position and provide evidence against self-renewal of MVCs; rather, they appear to differentiate from proliferating progenitor cells. These properties are compatible with a regulatory role in sensory neuron regeneration, which requires MVCs to be insensitive to stimuli that induce their apoptosis.

Keywords

Olfaction; neurogenesis; CD73; BrdU; neuronal homeostasis

Introduction

The nasal cavity is directly exposed to air-born contaminants and, as a consequence, cells in the nasal epithelia are prone to damage. Therefore, olfactory cells, including olfactory neurons and other chemosensory cells, such as solitary chemosensory cells (SCCs), have a short lifespan and get replaced from progenitor cells throughout adult life (Graziadei et al., 1978; Schwob, 2002; Gulbransen and Finger, 2005). In the olfactory epithelium (OE), the basal germinal zone, from which regeneration occurs, contains two distinct cell populations with stem cell characteristics, the globose (GBCs) and horizontal basal cells (HBCs) (Mackay-Sim and Kittel, 1991; Huard et al., 1998). GBCs are multipotent and have the highest proliferation rate in the OE (Caggiano et al., 1994; Goldstein et al., 1998; Jang et al., 2003; Chen et al., 2004). HBCs are postulated to represent relatively quiescent, multipotent progenitors. Recent studies have demonstrated that extensive injury of the neuroepithelium can induce proliferation of HBCs to replenish the pools of neuronal and non-neuronal cells (Packard et al.; Carter et al., 2004; Leung et al., 2007; Iwai et al., 2008). Interestingly, the supporting cells arranged in a single layer at the apical surface possess the capability to self-renew in the unperturbed OE. Nevertheless, after massive damage to the OE they get replaced from progenitor cells, as well (Schwob et al., 1995; Weiler and Farbman, 1998).

Besides these four cell types (olfactory neurons, supporting cells, GBCs and HBCs), several populations of microvilli-bearing cells have been described in rodent OE (Moran et al., 1982; Rowley et al., 1989; Carr et al., 1991; Braun and Zimmermann, 1998; Asan and Drenckhahn, 2005; Elsaesser et al., 2005; Hansen and Finger, 2008; Hegg et al., 2010). In previous work, we characterized one population of microvillous cells (Elsaesser et al., 2005; Montani et al., 2006), that was firstly described by Jourdan (Jourdan, 1975). These flask-shaped cells, named microvillar cells (MVCs), represent 5% of all olfactory cells and are clearly distinct from olfactory neurons. They are evenly distributed throughout the neuroepithelium and are

situated at the most superficial layer intermingled with supporting cells, and therefore are in close contact with the incoming air stream. Furthermore, MVCs are equipped with an inositol-triphosphate ($InsP_3$)–mediated signal transduction cascade, including phospholipase C beta2 (PLC β2), type-3 IP3-receptors (IP3R3) and TRPC6-channels. Hence, MVCs can may convert extracellular signals in a strong calcium response mediating the output of these cells (Elsaesser et al., 2005; Montani et al., 2006). Characterization of MVCs provided support for their probable role in detecting signals released by degenerating olfactory neurons and coordinating proliferation and differentiation of progenitor cells. MVCs express the neuroproliferative factor neuropeptide Y (NPY) (Hansel et al., 2001; Montani et al., 2006). NPY most likely regulates GBCs via NPY receptor type Y1 (Doyle et al., 2008). Furthermore, ATP, released into the extracellular space following injury, can initiate progenitor cell proliferation and differentiation via purinergic receptors (Hassenklover et al., 2009; Jia et al., 2009). Interestingly, the release of NPY is triggered by ATP (Kanekar et al., 2009; Jia et al., 2011) and ATP increases the expression of NPY in MVCs *in vivo* (Jia and Hegg, 2010). Therefore, MVCs could function as a signaling link between degenerating neurons and stem cells, thus contributing to the control of neural proliferation in the postnatal OE.

The continuous turnover of olfactory neurons and their replenishment after damage is of high importance to maintain the functional integrity of the system. Not only neurons undergo turnover in the OE, but most likely the majority of olfactory cells. So far, the question whether MVCs get replaced in the adult animal, and at which rate, is unresolved. As a corollary, it remains elusive whether they self-renew (like supporting cells) or arise from progenitor cells. To answer these questions, we monitored the fate of MVCs pulse-labeled with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) combined with immunofluorescence staining using a novel selective marker for MVCs, ecto-5'-nucleotidase

(CD73). CD73 is a glycosyl phosphatidylinositol (GPI-) linked, membrane bound glycoprotein that catalyzes the extracellular hydrolysis of 5'-AMP to adenosine, which may subsequently activate adenosine receptors (Zimmermann, 1992). Thereby, CD73 is involved in various physiological processes mediated by adenosine including hypoxia, inflammation, antinociception, epithelial ion transport, and modulation of the blood brain barrier functions (Koszalka et al., 2004; Thompson et al., 2004; Colgan et al., 2006; Mills et al., 2008; Niemela et al., 2008; Sowa et al., 2010a; Sowa et al., 2010b). CD73 has been described in the rat OE to label dark/horizontal cells at the basal side and microvillous cells at the luminal side (Braun and Zimmermann, 1998). Here, we establish CD73 in the mouse OE as an unequivocal marker for MVCs, notably by comparison with a transgenic mouse expressing eGFP driven by the IP3R3 promoter (Hegg et al., 2010).

Material and Methods

Animals

IP3R3$^{tm(tauGFP)}$ transgenic mice in which the first exon of the *Itpr3* gene is replaced by the coding region of the fusion protein tau-eGFP (C.C Hegg *et al.*, 2010), were kindly provided by Dr. Diego Restrepo (University of Colorado Denver, CO). Adult male IP3R3^{+}/IP3R3^{-} tauGFP^{+} mice were used for immunohistochemistry. Additionally, immunohistochemistry was performed in tissue from male C57BL/6J wild-type mice bred in our Institute. All experimental procedures were approved by the Cantonal Veterinary Office in Zurich. The mice were housed 3 – 6 animals per cage in a 12 h light/dark cycle with food and water provided ad libitum.

Mouse olfactory epithelium tissue preparation

Mice were given a single intraperitoneal injection of 180 mg/kg bromo-deoxyuridine (BrdU; Sigma-Aldrich, Switzerland, #B5002) dissolved in 0.9% NaCl with an injection volume of 6.6 ml/kg body weight. At various time spans after BrdU injection (1, 3, 5, 7, 10, 14, 21, 28 and 42 days), mice were deeply anesthetized with Nembutal (50 mg/kg, i.p.) and perfused transcardially with phosphate buffered saline (PBS) followed by aldehyde fixation (4% paraformaldehyde,15% saturated picric acid, 150 mM sodium phosphate buffer, pH 7.4). Thereafter, the noses were rapidly dissected and stored for 2 h in fixative at 4°C. After washing, they were decalcified in 5% ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 7 days at 4 °C. Next, the specimens were cryoprotected overnight in 30 % sucrose in PBS and subsequently frozen and stored at -80°C. The specimens were embedded with Neg-50 Frozen Section Medium (Richard-Allan scientific, MI) and coronal sections of 20 µm thickness were cut and mounted on gelatine-coated slides.

Whole-mount preparation: Animals were decapitated and the OE was dissected out and transferred into fixative. The tissue was fixed for one hour at 4°C. After washing, the tissue was processed for immunohistochemistry.

DNA denaturation for BrdU staining

The sections were air-dried before being washed in PBS and incubated in 0.2 N HCl at room temperature (RT) for 5 min. Thereafter, they were transferred into 4 N HCl and incubated at 37°C for 30 min. After denaturation, the sections were washed four times in PBS and processed for immunohistochemistry.

Immunofluorescence staining

The primary antibodies used are listed Table 1; they were diluted in PBS containing 5% normal serum and 0.2% Triton X-100. Thereafter, sections were incubated overnight at 4°C, washed three times in PBS for 10 min and incubated for 45 min at RT with secondary antibodies raised in goat or donkey and conjugated to Alexa488 (Molecular Probes) or Cy3 (Jackson Immunoresearch, West Grove, PA). They were diluted in PBS containing 5% normal serum (Alexa488 1:1000; Cy3 1:500). The sections were air-dried and coverslipped using DAKO fluorescence mounting medium (Dako North America, CA).

In order to detect CD73 and BrdU simultaneously, the immunofluorescence staining protocol was slightly adjusted. To this end, sections were incubated overnight at RT with rat-anti-CD73 antibody (1:1000) containing 5% normal serum and 0.2% Triton X-100. Following three 10-minutes washing steps, sections were incubated for 30 min at RT with biotinylated secondary antibody (1:500) diluted in PBS containing 5% normal serum and 0.02% Triton X-100. After washing, the slides were transferred into fixative for 12 min at 4°C. This was followed by three times 10 min washing in PBS. DNA denaturation was applied by incubating the sections firstly for 5 min in 0.2N HCl at RT and subsequently for 30 min in 4N HCl at 37°C. Next, sections were washed three times in PBS before incubating for 20 hours

at RT with FITC-conjugated anti-BrdU antibody (1:100) in PBS containing 5% NGS and 0.02% Triton X-100. Slides were rinsed in PBS and transferred into Cy3-conjugated streptavidin solution and incubated for 30 min at RT. Thereafter, they were washed, air-dried and coverslipped with DAKO mounting medium.

Image processing and analysis

Sections processed for immunofluorescence were analyzed by confocal microscopy (LSM-710, Zeiss, Jena, Germany) using 40x (NA 1.3) or 63x (NA 1.4) and sequential acquisition of separate channels. Z-stacks of consecutive sections (5-8; 1024 x 1024 pixel; spaced 1 µm in z) were acquired with the pinhole set at 1 Airy unit. For visual display, image stacks were projected in the z-dimension and merged using the image analysis software Imaris (Bitplane, Zurich, Switzerland).

Three animals per time span post BrdU-injection were used to quantify the number of MVCs and BrdU-positive MVCs, respectively. Sampling fields containing CD73-immunoreactive cells in the OE were selected. Two different areas were used to count the cells. The first area contained all ethmoid turbinates and the second area comprised the ethmoid turbinates, at the level of the rostral part of the olfactory bulb. Four sampling fields in each area were acquired using the confocal microscope with the 40x lens. Stacks of 11-12 confocal layers were projected in 2-D and used for cell counts. Cells were only considered as double-labeled if the $BrdU^+$ nucleus was clearly visible.

Statistical analysis was performed by Kruskal-Wallis test using GraphPad Prism (GraphPad Software, Inc. Version 4.01). Statistical significance was set at $p < 0.005$.

Results

CD73 is a selective marker for microvillar cells in the mouse OE

In previous studies, we investigated MVCs as a major microvilli-bearing cell type in the OE characterized by expression of a phosphatidyl-inositol-mediated signal transduction pathway and postulated their involvement in the control of neuronal proliferation in the postnatal OE(Elsaesser et al., 2005). Here, we detected the membrane-bound CD73 (ecto-5'-nucleotidase) as a novel marker for MVCs. Using anti-CD73 antibodies in whole-mount specimen of adult mice revealed immunoreactive cells that were evenly scattered throughout the sensory OE (Figure 1A). In double labeling experiments with CD73 and OMP, CD73-immunoreactive cells were never co-localized with knobs of ciliated neurons (Figure 1A). Staining of olfactory tissue sections demonstrated that anti-CD73 antibodies labeled cells that strongly resembled our previously described MVCs (Elsaesser et al., 2005) based on their density, shape and localization in the epithelium (Figure 1 B-D). In order to substantiate the selectivity of CD73 as a marker for MVCs, we used co-labeling of CD73 with an element of the phosphatidyl-inositide signaling cascade. Double staining with antibodies against CD73 and PLC β2 showed that these two proteins were always co-associated within the same cell (Figure 1B). To ensure that CD73 is selectively expressed by MVCs, we examined two markers for olfactory neurons, OMP and AC-III. Neither at the apical pole CD73 was co-localized with OMP or AC-III, nor at the basal process did CD73 overlap with OMP (Figure 1C). CD73 was detected noticeably underneath the layer of AC-III-positive cilia (Figure 1C''). In conclusion, our data indicate that CD73 can be used as a reliable marker for MVCs.

IP3R3-eGFP microvillous cells are immunoreactive for CD73

Several types of microvillous cells have been reported in the OE. However, the role of microvilli-bearing cells is only poorly understood, and their classification into various types remains to be elucidated. Recently, Hegg and colleagues (Hegg et al., 2010) described microvillous cells expressing type-3 IP3-receptors using an $IP3R3^{tm1(tauGFP)}$ transgenic mouse strain. This cell type has several features in common with MVCs. Both of them bear microvilli at their apical protrusions, they possess axon-like processes that do not penetrate the basal lamina, and they do not degenerate after bulbectomy. Furthermore, they respond to odors with an increase of intracellular Ca^{2+} and do not express neuronal markers. Hence, both of these microvillous cell types were classified as non-neuronal chemoresponsive cells (Elsaesser et al., 2005; Hegg et al., 2010). In order to clarify whether they represent the same cell type, we examined CD73 immunoreactivity in $IP3R3^{+}/IP3R3^{-}$ $tauGFP^{+}$ mice. Whole-mount preparations revealed that every IP3R3-tauGFP-positive cell was capped by CD73 immunostaining (Figure 2A). Likewise, in sections we detected that CD73-immunofluorescence was selectively co-associated with IP3R3-tauGFP-positive cells (Figure 2B). Furthermore, we used anti-PLC β2 antibodies to test the distribution of an additional marker of MVCs in IP3R3-tauGFP-positive cells. As expected, PLC β2 immunoreactivity was present in virtually all IP3R3-tauGFP-positive cells in whole mount preparation and in tissue sections (Figure 2C-D). Therefore, these results indicate that these two populations of microvillous cells (MVCs and IP3R3 MV cells) correspond to the same cell type.

MVC turnover

There are many open questions about the role and regulation of MVCs in the postnatal OE. In particular, it is not known whether differentiated MVCs undergo cell division and thereby self-renew or whether they originate from a population of olfactory progenitor cells. Having CD73 as an excellent marker to selectively detect MVC, we next determined the turnover of

MVCs. To this end, we applied an immunohistochemical protocol allowing the simultaneous detection of MVCs and newly born cells in the same tissue section. Mice were injected once with BrdU and the proportion of BrdU-labeled MVCs at early and late time-points post-injection was compared. As expected, many cells were immunoreactive for either CD73 or BrdU and only a small percentage was double-labeled. At 1 dpi, BrdU immunoreactivity was mainly visible in the basal germinative zone. The majority of BrdU-positive cells in this region constitute the progenitor cell population (Figure 3A-B). Nevertheless, there were also a few BrdU-immunoreactive cells detectable at the most superficial layer of the epithelium, presumably representing self-renewing supporting cells. Upon cell division, supporting cells often display a typical arrangement with two neighbouring nuclei being labeled, because the mother and daughter cell remain adjacent to each other (Figure 3B). However, there were no BrdU/CD73 double-labeled cells detectable at 1 dpi. The first BrdU-positive MVCs were observed at 3 dpi. Thus, a cell requires 3 days between S-phase and the earliest detectable expression of CD73. Double-labeled cells were observed more frequently at 5, 7, 10 dpi (Figure 3C-D). These results indicate that MVCs are replaced in the adult animal. At 10 dpi the number of BrdU-positive MVCs increased to a maximum with a mean value of 2.5% MVCs being double-labeled at this time point. Later on the number of BrdU-labeled MVCs gradually decreased to 0.5 % at 21 dpi. But noticeably, when increasing BrdU to two injections per day for 3 days BrdU-positive MVCs were detectable even at later time points after injection (21, 28, 42 dpi). This finding together with the observation that a relatively low percentage of MVCs were BrdU-positive at all time points, reaching from 0.5% to a maximum of 2.5%, indicates that MVCs represent a stable cell population with a long lifespan. Quantification of the numerical density revealed a constant number of MVCs over the time period investigated. In line, Kruskal-Wallis analysis revealed no statistical effect of time for the number of MVCs in the OE ($n = 3$ per time point; $P = 0.0903$; $H = 10.939$). As

expected from the monitoring of BrdU-labeled MVCs, Kruskal-Wallis analysis yielded a significant overall time effect of the proportion of BrdU-positive MVCs (n = 3 per time point; P = 0.008; H = 17.126; Figure 3E). Taken together, we determined that MVCs are replaced in the adult animal. However, they most likely do not have the ability to self-renew, but are rather replenished by stem cells.

Discussion

Multiple subtypes of microvillous cells have been described in the OE. They are distinct with regard to the morphological description and their proposed functions were often controversially described. MVCs represent one population of microvilli-bearing cells that might contribute to the control of neuronal proliferation in the postnatal OE (Elsaesser et al., 2005; Montani et al., 2006). In a recent study, Hegg and colleagues reported an IP3R3 expressing microvillous cell population (IP3R3 MV cells) using $IP3R3^{+}/IP3R3^{-}$ $tauGFP^{+}$ mice (Hegg et al., 2010). This cell population strongly resembled MVCs described by our group. Hegg *et al.* suggested that MVCs might be a subset of these IP3R3 MV cells based on the failure of PLC β2 staining and the observation of only some TRPC6-immunoreactive cells among GFP^{+} MV cells (Hegg et al., 2010). Here, we firstly established CD73 as a reliable and selective marker for MVCs and accordingly demonstrated CD73 expression in IP3R3 microvillous cells. Moreover, an additional marker for MVCs, PLC β2 was co-associated with IP3R3 using co-immunofluorescence staining employing tissue section of $IP3R3^{+}/IP3R3^{-}$ $tauGFP^{+}$ mice. For these reasons, we suggest that MVCs and IP3R3 MV cells constitute very likely the same microvillous cell population. This cell population is endowed with multiple elements of the $InsP_3$–mediated signal transduction cascade, including IP3R3, PLC β2, Gq/11 and TRPC6. In addition, they have the ability to convert various extracellular signals, such as odorants, ATP and substance P, in a strong calcium response (Elsaesser et al., 2005; Hegg et al., 2010). However, one can exclude this microvillous cell population (MVCs and IP3R3 MV cells) to correspond to TrpM5-positive microvillous cells described by Hansen and Finger, since TrpM5-positive cells display a different morphology and do not express PLC β2, TRPC6 and IP3R3 (Hansen and Finger, 2008). Further studies will be needed, however, to elucidate the detailed classification and specific role of each microvillous cell subpopulation and determine possible species differences.

Adult stem cells are present in many tissues and have the ability to replace a majority of somatic cells. Several types of somatic cells that are exposed to noxious stimuli undergo a lifelong cycle of cell death and replacement. In the OE, the germinative zone lies adjacent to the basal lamina and contains multipotent cells. Olfactory cells, including sensory neurons, are continuously restored throughout life. The present study shows that also MVCs get replaced. We detected the earliest BrdU-labeled MVCs at 3 dpi and observed a peak of newly generated MVCs at 10 dpi. This temporal profile is very similar to the maturation of other sensory cells. For example, olfactory sensory neurons need approximately one week to mature. BrdU-OMP double labeled cells were reported to appear 7 days after BrdU injection (Miragall and Monti Graziadei, 1982; Schwob et al., 1992; Kondo et al., 2010). Solitary chemosensory cells lying in the non-olfactory nasal epithelium express elements of their transduction cascade, α-gustducin, within 3 days after completing mitosis (Gulbransen and Finger, 2005). In Type II taste vallate bud cells, α-gustducin and BrdU were co-expressed starting from 2.5 dpi and reaching a peak at 6.5 dpi (Farbman, 1980; Cho et al., 1998). The signaling molecule for taste transduction, PLC β2, has been shown to be expressed in taste bud cells from day 5 and reached maximum at day 12 (Hamamichi et al., 2006). The timing of CD73 expression in MVCs after three days exiting S-phase indicates that MVCs mature at a rate similar to other sensory cells.

Interestingly, at 10 dpi the proportion of BrdU-labeled MVCs started to decline, indicating degeneration of some newly generated MVCs. This observation is again in line with previous reports. The lifespan of taste bud cells shows a similar pattern, with roughly half of the newly incorporated cells being eliminated within the first few days after birth (Hamamichi et al., 2006). The number of newly generated olfactory sensory neurons that express OMP increases gradually from 7 days to 14 days and thereafter starts to decrease. Moreover, a large fraction of BrdU-positive cells underneath the OMP-positive cell layer dies within 14 days after birth

(Kondo et al., 2010). One explanation of the decrease in BrdU-labeled MVCs might be that newly produced MVCs are vulnerable to the exposure of noxious stimuli and/or they need to receive trophic support for their survival. Olfactory sensory neurons, for instance, depend on signals from the olfactory bulb and from locally released factors within the OE (Mackay-Sim and Kittel, 1991; Schwob et al., 1992; Carr and Farbman, 1993; Kondo et al., 2010).

It is the current understanding that the degree of exposure of a cell type to noxious stimuli is loosely correlated with its turnover; for example, airway epithelial cells lining the tracheo-bronchial tree have a significant longer turnover than cells in the nasal cavity (Basbaum and Jany, 1990). Consequently, SCCs, located in a remarkably vulnerable location at the anterior end of the nasal cavity, have an estimated turnover of only 20 days (Gulbransen and Finger, 2005). Likewise, olfactory sensory neurons in a slightly more protected position at the posterior part of the nasal cavity have a lifespan of one to two months (Moulton, 1974; Graziadei and Graziadei, 1979; Miragall and Monti Graziadei, 1982; Mackay-Sim and Kittel, 1991; Schwob et al., 1992; Schwob, 2002). Exceptions are the olfactory supporting cells, involved in forming the apical layer of the OE, which are considered to have a long lifespan and be replaced at low rate (Naguro and Iwashita, 1992; Weiler and Farbman, 1998). Since MVCs are intermingled with the supporting cells at the most superficial layer in close contact with the incoming airstream, they are prone to cellular damage, pointing to a short turnover. However, we detected double-labeled cells occurring with a remarkably low frequency indicating that MVCs, like their neighbouring supporting cells, have a slow turnover and a long lifespan. Even at 42 dpi there were BrdU-labeled MVCs detectable. These properties are in accordance with their proposed function to be involved in regulating neurogenesis, since this role requires MVCs to represent a stable cell population that is insensitive to pro-apoptotic stimuli.

The majority of epithelial cells is generated from a population of undifferentiated progenitor cells (Chen et al., 2004). These progenitor cells have the capacity to replace cells and maintain the epithelial homeostasis. Olfactory neurons are amongst the best-studied example of how regeneration from stem cells occurs. The cell lineage relationship has been extensively described; starting from undifferentiated precursor cell types to mature neurons. Evidence indicates that olfactory neurons are generated by GBCs, at least in undamaged tissue (Caggiano et al., 1994; Huard et al., 1998). As one exception, supporting cells have the capacity to proliferate and can replace themselves (Graziadei and Graziadei, 1979; Weiler and Farbman, 1998). Our data indicates that unlike the supporting cells, differentiated MVCs do not divide and thus, MVCs rather derive from a population of olfactory progenitor cells. The identity of this progenitor cell remains elusive. MVCs might originate from GBCs, as olfactory neurons. Other likely candidates are the HBCs, since they display a rather quiescent dividing characteristic, in keeping with the rare occurrence of BrdU-labeled MVCs. As a third possibility, a distinct progenitor cell population might give rise to MVCs. Further investigations are required to clarify the origin of MVCs.

Altogether, our results show that one microvilli-bearing cell type, the MVCs, undergo turnover and are, therefore, similar to the majority of somatic cells that can be replaced throughout life, most likely to protect the organism against damage. Because of the postulated involvement of MVCs in olfactory neurogenesis, it will be essential to further investigate their function and their own regulation for a better understanding of the molecular and cellular pathways underlying progenitor cell proliferation and differentiation.

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Abbreviations

ACIII, adenylyl cyclase III; ATP, adenosine-5'-triphosphate; BrdU, 5-bromo 2'-deoxy-uridine; CD73, ecto-5'-nucleotidase; CNS, central nervous system; dpi, days post injection; EDTA, ethylenediamine tetraacetic acid ; GBC, globose basal cell; HBC, horizontal basal cell; $InsP_3$, inositol-triphosphate; IP3R3, inositol triphosphate receptor type 3; MV, microvillous; MVC, microvillar cell; NPY, neuropeptide Y; OE, olfactory epithelium; OMP, olfactory marker protein; PBS, phosphate buffered saline; PFA, paraformaldehyde; PLC β2, phospholipase C β2; RT, room temperature; SCC, solitary chemosensory cell; TRPC6, transient receptor potential channel 6.

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Figure Legends

Figure 1 CD73 as a marker of microvillar cells in the olfactory epithelium. (A) Double immunofluorescence staining on whole mount tissue preparations using anti-CD73 antibody (red) and anti-OMP antibody (green) revealed two separate cell populations, microvillar cells and olfactory sensory neurons. (B) Double immunofluorescence labeling against CD73 (red) and PLC β2 (green) on coronal sections stained the same cells. (C) Triple staining on coronal sections using anti-CD73 antibody (red), anti-OMP antibody (green) and anti-ACIII antibody (blue). Neither OMP-immunoreactive receptor neurons were co-associated with CD73-positive cells (C') nor did ACIII overlap with CD73 on cilia (C''). Scale bars: 20 μm (A), 10 μm (B-D).

Figure 2 Identification of IP3R3-GFP positive cells as microvillar cells. Immunofluorescence staining was performed on tissue sections from $IP3R3^{+}/IP3R3^{-}$ $tauGFP^{+}$ mice. (A-B) CD73-immunoreactivity (red) is detected in IP3R3-GFP-positive cells. (A) Whole mount tissue preparations; (B) coronal section of the olfactory epithelium. (C-D) Immunofluorescence staining using anti-PLC β2 antibody revealed co-association with IP3R3-GFP microvillous cells in whole mount tissue (C) and coronal sections (D). Scale bars: 20 μm (A), 10 μm (B-D).

Figure 3 BrdU pulse-chase labeling combined with immunohistochemical staining for CD73 provides evidence for microvillar cell turnover. (A-D) Double immunofluorescence staining on coronal sections using anti-CD73 antibody (red) and anti-BrdU antibody (green). (A) At 1 dpi, most of the BrdU-positive cells are present at the basal part of the olfactory epithelium. (B) At 3 dpi, BrdU labeled cells are migrating apically indicating differentiating cells. Arrow shows self-renewing supporting cells. (C-D) BrdU labeled MVCs are detectable at 5 dpi (C) and 10 dpi (D). (C') Magnification of C. (E) Fraction of microvillar cells double-labeled for

BrdU and CD73 at different time points after BrdU injection. The first double-labeled cells appear at 3 dpi. The percentage of BrdU-labeled microvillar cells gradually increased peaked at 10 dpi and thereafter declined. Kruskal-Wallis test confirmed a statistical effect of time (N = 3 for time point; P = 0.008; H = 17.126). Each dot represents the value from an individual animal. Scale bars: 10 μm.

Table 1 List of antibodies

Antibody	Specificity	Manufacturer	Description/Nr	Dilution
ACIII	(Wei et al., 1998; Wong et al., 2000)	Santa Cruz Biotechnology, Inc., USA	Rabbit polyclonal; #sc-588	1:1000
BrdU: FITC	(Vanderlaan and Thomas, 1985; Kondo et al., 2010)	AbD Serotec, Oxford, UK	Rat monoclonal, IgG2a conjugated to FITC-liquid; #OBT0030F	1:100
CD73	(Yamashita et al., 1998; Eliopoulos et al., 2005; Kobie et al., 2006)	eBioscience, Inc; San Diego, USA	Rat monoclonal, IgG1; #16-0731	1:1000-1:1500
IP3R3	(Blondel et al., 1993; Leite et al., 2003)	BD Bioscience, USA	Mouse monoclonal, IgG2a, #610312	1:500
OMP	(Baker et al., 1989; Cummings et al., 2000)	Wako, Chemicals, Richmond, USA	Goat polyclonal, #544-10001	1:500
PLC β2	(Ali et al., 1997; Perez et al., 2002)	Santa Cruz Biotechnology, Inc., USA	Rabbit polyclonal, Q-15, #sc-206	1:500

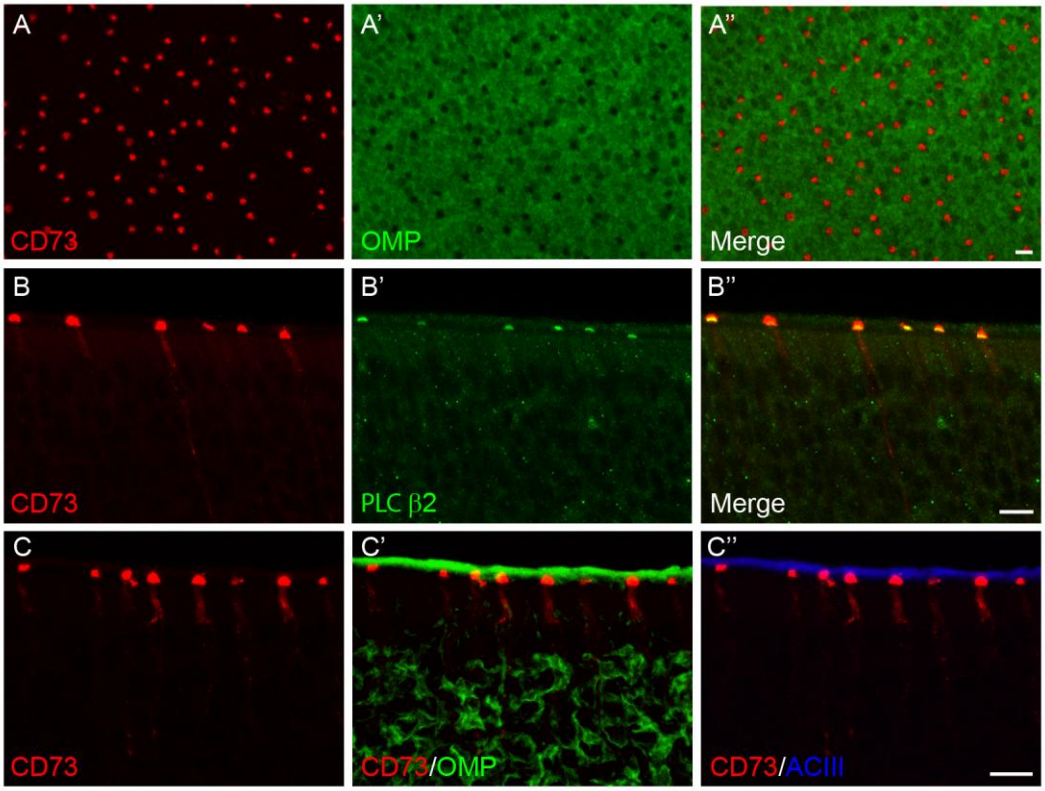
A
CD73
A'
OMP
A''
Merge
B
CD73
B'
PLC β2
B''
Merge
C
CD73
C'
CD73/OMP
C''
CD73/ACIII

Figure 1

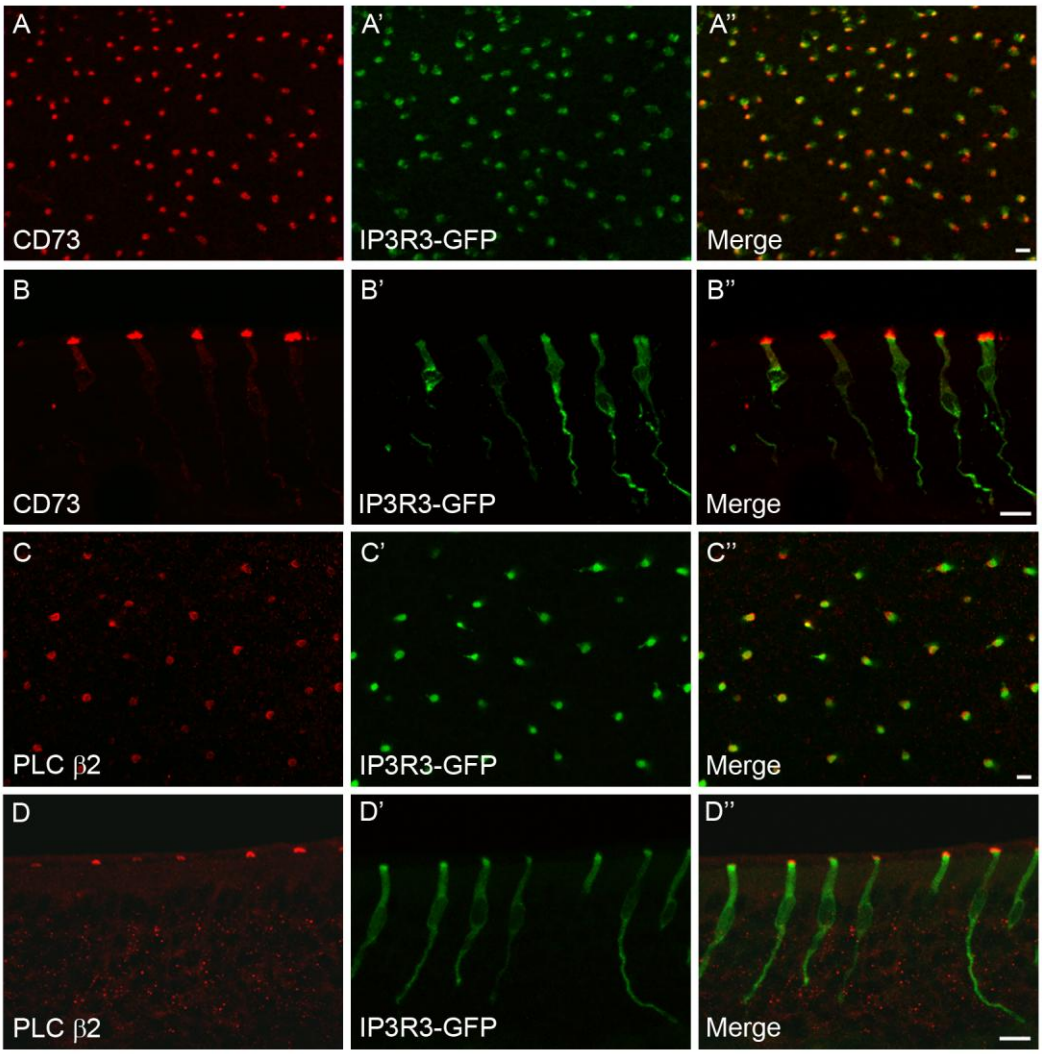
A
CD73
A'
IP3R3-GFP
A''
Merge
B
CD73
B'
IP3R3-GFP
B''
Merge
C
PLC β2
C'
IP3R3-GFP
C''
Merge
D
PLC β2
D'
IP3R3-GFP
D''
Merge

Figure 2

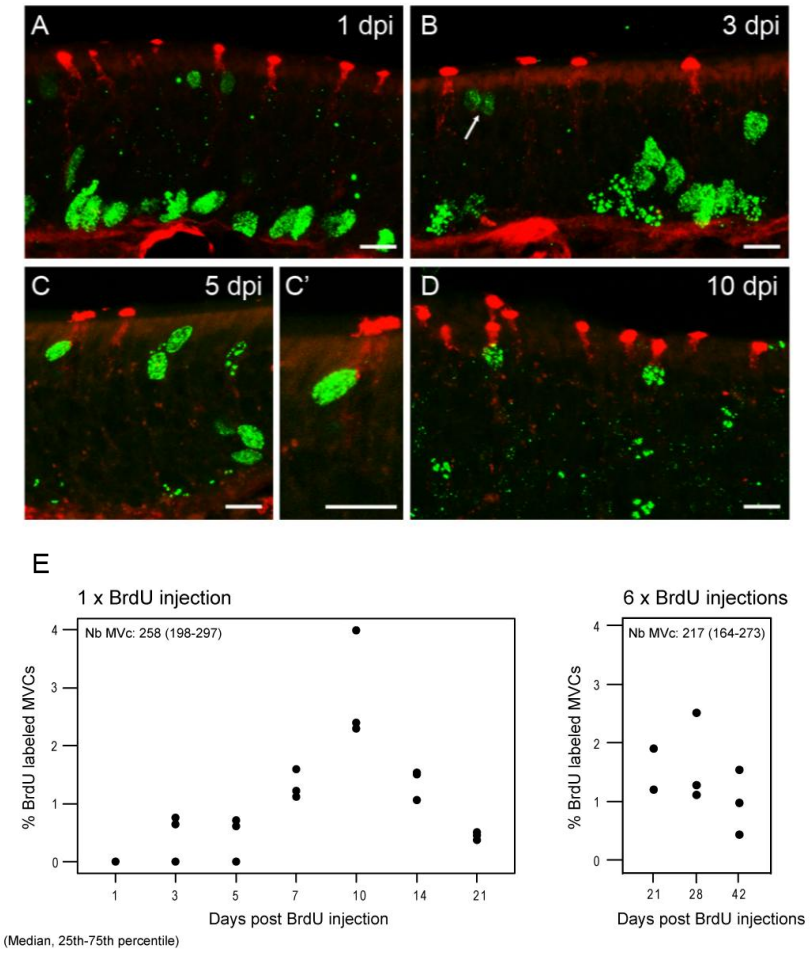
A
1 dpi
B
3 dpi
C
5 dpi
C'
D
10 dpi
E
1 x BrdU injection
Nb MVc: 258 (198-297)
% BrdU labeled MVCs
Days post BrdU injection
(Median, 25th-75th percentile)
6 x BrdU injections
Nb MVc: 217 (164-273)
% BrdU labeled MVCs
Days post BrdU injections

Figure 3